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Heterologous expression of a *Photorhabdus luminescens* syrbactin-like gene cluster results in production of the potent proteasome inhibitor glidobactin A

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Abstract

Syrbactins are cyclic peptide derivatives which are known to inhibit the eukaryotic proteasome by irreversible covalent binding to its catalytic sites. The only two members of this family characterized to date, syringolin A and glidobactin A, are secreted by certain strains of *Pseudomonas syringae* pv. *syringae* and strain K481-B101 from the order *Burkholderiales*, respectively. Syrbactins are the products of mixed non-ribosomal peptide/polyketide synthases encoded by gene clusters with a characteristic architecture. Similar, but not identical gene clusters are present in several other bacterial genomes, including that of *Photorhabdus luminescens* subsp. *laumondii* TT01, which is therefore hypothesized to be able to produce a syrbactin-type proteasome inhibitor. Here we report the cloning of the putative syrbactins synthetase encoding gene cluster of *Ph. luminescens* into a cosmid vector and its heterologous expression in *Pseudomonas putida.* Analysis of culture supernatants of transformed *Ps. putida* by HPLC and mass spectrometry revealed the presence of glidobactin A, indicating that the syrbactins-like gene cluster of *Ph. luminescens* encodes a glidobactin A synthetase and that this organism has the capacity to synthesize glidobactin A.

Introduction

Syrbactins are a structural class of potent proteasome inhibitors that covalently and irreversibly bind to the active site threonine residues of catalytic proteasome subunits (Groll et al., 2008; Krahn et al., 2011). Syringolin A (Figure 1), one of the two well characterized syrbactins to date, is secreted by strains of the phytopathogenic bacterium *Ps. syringae* pv. *syringae* (Wäspi et al., 1998). It is the major variant of a small family of closely related compounds thought to be the products of the same

synthetase (Wäspi et al., 1999), which is a mixed non-ribosomal peptide synthetase/polyketide synthetase (NRPS/PKS) encoded by the *sylA-sylE* gene cluster (Amrein et al., 2004). Analysis of the gene cluster, in particular of the architecture of the *sylC* and *sylD* genes, have allowed to postulate a biosynthesis model which completely explains the tripeptide part containing the ring structure (Amrein et al., 2004). Recently, other experiments also shed light on the biosynthesis of the tail part of syringolin A, including its unusual ureido group (Imker et al., 2009; Ramel et al., 2009; Wuest et al., 2011). Glidobactin A, the other well characterized syrbactin member, has a twelve-membered ringstructure similar to the one of syringolin A, but exhibits an acylated tail (Figure 1A). It is secreted by an unknown species belonging to the order *Burkholderiales* and is the product of a synthetase encoded by the *glbA-glbH* gene cluster, which is homologous to the syringolin gene cluster (Figure 1B) (Schellenberg et al., 2007). Like in the case of syringolin A, the architecture of the *sylC* and *sylD* homologs *glbF* and *glbC*, suggested a glidobactin A biosynthesis model which completely explains the twelve-membered ring structure with the exception of the hydroxyl group (Schellenberg et al., 2007). Recently, the acylation of the N-terminal threonine residue has been shown to be mediated by the *glbF*gene product, which, in addition to activating the threonine residue, also mediates its acylation using acyl-CoA as a donor (Imker et al., 2010). Glidobactin A is the major variant of a family of compounds also named cepafungins that differ mostly in the structure of the fatty acid tail (Oka et al., 1988a; Oka et al., 1988b; Terui et al., 1990).

Among the bacteria whose genomes have been completely sequenced, sequence comparison revealed gene clusters similar to *glbA-glbH* in the insect pathogen *Photorhabdus luminescens* as well as in the human pathogens *Photorhabdus asymbiotica, Burkholderia pseudomallei* (causing agent of melioidosis), and *B. oklahomensis*, while *B. mallei* strains contain a disrupted cluster (Schellenberg et al., 2007). In comparison to the *glb* gene cluster, these organisms lack *glbA* and *glbH* homologues, and in some cases also a *glbE* and a *glbG* homologue (Figure 1B). While *glbA*

encodes a LysR-type transcription factor and *glbE* an MbtH-like peptide, both of which are irrelevant for structural considerations (Lautru et al., 2007; Rackham et al., 2010; Schellenberg et al., 2007; Zhang et al., 2010), the functions of *glbG* and *glbH* are hitherto unknown.

We were interested to compare the structure of the *Ph. luminescens* syrbactins-like gene cluster, which encompasses genes *plu1881-1877*, and the structure of the product of the synthetase encoded by these genes in order to improve our ability to derive product structure from the genes and their architecture. *Plu1881-1877*are homologous to *glbB-glbG*, except that the MbtH-like protein encoded by *glbE* is missing (Figure 1B). However, all our efforts to isolate putative syrbactins from *Ph. luminescens* spp. *laumondii* (*Pll*) TT01 grown under a variety of conditions failed. Thus, we decided to clone *plu1881-1877* and express the gene cluster in a heterologous system. Here we show that *Ps. putida* harboring a cosmid-borne *plu1881-1877* gene cluster secretes *bona fide* glidobactin A, suggesting the *Pll* is capable to synthesize this potent proteasome inhibitor.

Materials and methods

Bacterial strains and culture conditions

Unless stated otherwise, *Escherichia coli* strains, *Ps. putida* P3 (Senior et al., 1976), and *Ph. luminescens* spp. *laumondii* TT01 (accession no. DSM 15139, German Collection of Microorganisms and Cell Cultures (DSMZ,)) were grown in LB medium on a shaker at 220 rpm at 37° C, 28° C, or 30 ° C, respectively.

Cloning of the *plu1881-1877* gene cluster and transfer to *P. putida* P3

If not stated otherwise, standard procedures were used (Ausubel et al., 1987). Genomic DNA of *P. luminescence* subsp. *laumondii* TT01 was isolated using Genomic tip-100/G columns (Qiagen, Hilden, Germany) according to instructions of the manufacturer. Ten µg of genomic DNA was digested with the locally unique restriction enzymes SalI and XbaI (positions 2242496 and 2216847, respectively; accession no. NC_005126), separated on a 0.5% agarose gel at 4° C, blotted onto a nylon membrane, and hybridized to a ^{32}P-labeled probe amplified from *plu1880* with primers plu1880_f and plu1880_r (Table 2) in order to verify the expected fragment size. DNA fragments larger than 20 kp were cut out of an identical gel, purified as described (Ramel et al., 2009), and cloned into HindIII/BamHI digested broad-host range cosmid pLAFR3 (Staskawicz et al., 1987) with the help of adapters obtained by annealing oligonucleotides Hind-Sal_Adapt_H/ Hind-Sal-Adapt-S and Xba-Bam-Adapt-X/Xba-Bam-Adapt-B, respectively (Table 2). After the adapters were ligated to the digested vector at 4° C overnight, the modified vector was separated from oligonucleotides by agarose gel electrophoresis and purified using Wizard® *SV* Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Isolated genomic DNA fragments (>20 kb) were then ligated with the modified vector for two days at 4^{o} C. The products were concentrated by ethanol precipitation and packaged into λ-phage particles using Gigapack III Plus Packaging Extract (Agilent Technologies, Basel, Switzerland). The library was plated on *E. coli* XL-1 Blue MRA (Stratagene, La Jolla, California) and screened according to instructions of the manufacturer using the ^{32}P-labeled *plu1880* probe described above. Positive clones were isolated and completeness of the *plu1881-1877* gene cluster was verified by PCR using primers amplifying the left and right end of the cluster (primers plu1881_f/ plu1881_r and plu1877_f/plu1877_r, respectively; Table 2). The recombinant cosmid was named pL3plu and transferred from *E. coli* XL-1 Blue MRA to

Ps. putida P3 by triparental mating using the *E. coli* helper strain HB101 (pRK600) (Christensen et al., 1999).

Preparation and analysis of methanolic extracts

Five mL SRM_{AF} medium (Mo and Gross, 1991) containing 10 µg/mL tetracycline were inoculated with *Ps. putida* and shaken at 220 rpm over night at 28° C. The culture was used to inoculate 100 mL SRM_{AF} medium. These cultures were grown at 28° C either for 2 days at 220 rpm (shaken cultures), or without agitation for 5 days (still cultures). After centrifugation at 10 000 x g for 10 min, pelleted cells were resuspended in 90% HPLC-grade methanol and shaken at 250 rpm for 2 hours at room temperature. After centrifugation at 10 000 x g for 15 min, supernatants were filtered through 0.22 µm Steritop filters (Millipore, Molsheim, France) and stored at +4°C until used.

HPLC analysis was performed as described previously (Titus and Roundy, 1990) with some modifications. Extracts were concentrated 10-fold by complete evaporation and dissolution in 50% methanol followed by filtration through Millex GP filter units (0.22 µm pore size; Millipore, Molsheim, France). Twenty µL of filtrate was injected into a 250 mm x 4.6 mm Hypersil ODS 5 µm column (Dr. Maisch GmbH, Ammerbuch, Germany) which was connected to UltiMate 3000 HPLC system (Thermo Fisher, Olten, Switzerland). Equilibration of the column and isocratic separation of components was performed with a methanol:water (3:1) solution at a flow rate of 1 mL/min.

Methanolic extracts (50% methanol) were diluted 10:1 or 100:1 with CH_3CN and analyzed with a Waters Acquity UPLC system (Waters, Milford, USA) connected to a Bruker maXis QTof high-resolution mass spectrometer (Bruker Daltonics, Bremen, Germany). An Acquity BEH C18 HPLC column (1.7 µm, 2.1x100 mm fitted with a 2x2 mm guard column) has been used with a mixture of H_2O + 0.1% HCOOH (A) and CH_3CN + 0.1% HCOOH (B) solvent (0.3 ml flow rate,

linear gradient from 70 to 100% B within 3 min followed by flushing with 100% B for 1 min). The mass spectrometer was operated in the positive electrospray ionization mode at 4,000 V capillary voltage, -500 V endplate offset, with a N_2 nebulizer pressure of 1.6 bar and dry gas flow of 8 l min^{-1} at 200°C. MS acquisitions were performed in the mass range from *m/z* 50 to 1,200 at 20,000 resolution (full width at half maximum) and 1.5 scan s^{-1}. Masses were calibrated below 2 ppm accuracy with a 2 mM solution of sodium formate over *m/z* 158 up to 1450 mass range prior to analysis. The presence of Glidobactin was confirmed by calculation of extracted ion chromatograms of the $[M + H]^+$ signal at *m/z* 521.3333±0.05.

Results

In order to identify compounds produced by the *plu1881-1877*-encoded enzymes, a heterologous expression system was explored using *Ps. putida* strain P3, which has been successfully employed previously for the expression of the syringolin synthase cluster (*syl*) (Ramel et al., 2009). Analysis of the *Pll* TT01 genomic region containing *plu1881-1877* revealed unique SalI and Xba I restriction sites, with the SalI site being 2.54 kb upstream of *plu1881* and the XbaI located 4.77 kb downstream of *plu1877*. These restriction sites, which delimit a 25.65 kb fragment, completely contain *plu1881-1877*. The presence and uniqueness of these sites was confirmed by DNA gel blot analysis of SalI/XbaI-digested genomic DNA of *Pll* TT01 using a *plu1880*-derived hybridization probe (data not shown). A sub-genomic library was then prepared from SalI/XbaI-digested genomic DNA which was size-fractionated by separation on an agarose gel. Fragments larger than 20 kb were eluted and cloned into the broad-host range cosmid vector pLAFR3, packaged into λ-phage particles, and transfected into *E. coli*. The library was screened by hybridization with a *plu1880*-derived probe.

Positive colonies were isolated and presence of the entire cluster was the verified by PCR using primers amplifying the 5'-end of *plu1881* and 3'-end of *plu1877*. The recombinant cosmids of positive clones were transferred into *Ps. putida* P3. Presence of the cluster in transconjugant candidates was verified by PCR using *plu1881*, *plu1880* and *plu1877*-derived primers. Metabolite production was then tested by growing bacteria in a minimal medium either under constant shaking for two days, or as still cultures for five days. Methanolic extracts of pelleted cells were prepared and analyzed by HPLC. Comparison of HPLC profiles from transformed bacteria carrying the *plu1881-1877* gene cluster with profiles from the untransformed control strain reproducibly revealed a differential peak when cultures were grown under still culture conditions. As shown in the Figure 2, conditioned media of the transformed *P. putida* strain revealed a peak with a retention time identical to the one of isolated glidobactin A (8.7 min). LC-HR-ESI-MS of extracts revealed a quasi-molecular ion with an *m/z* of 521.33327, corresponding to a formula of $C_{27}H_{45}N_4O_6$ (hydrogen adduct, *m/z* 521.33336, SD of 0.18 ppm), which is identical to the one of the hydrogen adduct of glidobactin A (Figure 3). Thus, we conclude that the product of the NRPS/PKS encoded by the *plu1881-1877* gene cluster of *Pll* TT01 heterologously expressed in *Ps. putida* is glidobactin A, and that *Pll TT01* is capable to produce this potent proteasome inhibitor.

Discussion

We were hitherto unable to detect glidobactin A or similar compounds in *Pll* cultures grown in various media and conditions either by HPLC or by proteasome inhibition assays (unpublished results). Conditions explored included still and shaken cultures in minimal and insect culture media supplemented with insect hemolymph or proline, ingredients which have been shown to activate

expression of virulence-associated genes in *Pll* TT01 (Crawford et al., 2010). While these compounds were indeed able to slightly induce expression of the cluster, the level of production was apparently still below detection limits (data not shown). It is a notorious problem that for many NRPS and PKS gene clusters identified by genome sequencing, the products synthesized by the encoded enzymes remain unknown because these genes are not expressed under the culture conditions used or explored. In contrast to the glidobactin A gene cluster of K481-B101, the *Pll* syrbactin gene cluster does not encompass a transcriptional activator gene, suggesting that a regulator encoded elsewhere in the genome is involved. As to why the *Pll* gene cluster is active in *P. putida*, we can only speculate. One possibility is that the *Pll* gene cluster is activated in this organism by a regulator active under the culture conditions employed. *Pll* may also harbor a repressor of the syrbactin gene cluster that is absent in *P. putida*. In addition, the fact that multiple copies of the cosmid carrying the *Pll* gene cluster likely are present may also play a role.

Comparison of the amino acid residues forming the binding pocket of the NRPS modules encoded by *plu1880* and *plu1878* with the ones of their homologues of the glidobactin A producing strain K481-B101 suggests that the former activate the same amino acids as the modules present in GlbC and GlbF, respectively (Table 1). However, the detection of *bona fide* glidobactin A in culture supernatants of *P. putida* carrying the *plu1881-1877* gene cluster was unexpected because the gene cluster lacks a *glbH* homologue. The product of this gene is hypothesized to play a role in features of glidobactin A left unexplained by the biosynthesis model (Imker et al., 2010; Schellenberg et al., 2007), *i.e*. in the hydroxylation of the lysine residue, or in the modifications exhibited by the minor variants, which are all in the fatty acid tail with the exception of glidobactin G, where a hydroxymethyl group replaces the methyl group attached to the ring structure (Oka et al., 1988a; Oka et al., 1988b; Terui et al., 1990). Because *glbH* was not needed for glidobactin A biosynthesis in *Ps. putida* carrying *plu1881-1877*, *glbH* would seem to play a role in a modification leading to a

minor variant not present in *Pll*, and not in the hydroxylation of the lysine residue. Of course, it cannot be completely excluded that a gene in the *Ps. putida* genome substitutes for a *glbH* homologue, although BLAST analyses of the completely sequenced *Ps. putida* (and *Pll*) strains revealed no close homologue.

The *plu1881-1877* gene cluster also lacks a *glbE* homologue, which encodes an MbtH-like peptide. However, it has been shown that MbtH proteins encoded by different NRPS clusters are able to substitute for each other (Felnagle et al., 2010; Zhang et al., 2010), and BLAST analysis reveals that both *Pll* and *Ps. putida* contain at least one MbtH-encoding gene in other NRPS gene clusters which may provide the function if needed.

References

Amrein H, Makart S, Granado J, Shakya R, Schneider-Pokorny J, and Dudler R. Functional analysis of genes involved in the synthesis of syringolin A by *Pseudomonas syringae* pv. *syringae* B301D-R. Mol Plant-Microbe Interact 2004;17:90-97.

Ansari MZ, Yadav G, Gokhale RS, and Mohanty D. NRPS-PKS: a knowledge-based resource for analysis of NRPS/PKS megasynthases. Nucleic Acids Res 2004;32:W405-W413.

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, and Struhl K. Current protocols in molecular biology. New York: Wiley and Sons; 1987.

Christensen BB, Sternberg C, Andersen JB, Palmer RJ, Nielsen AT, Givskov M, and Molin S. Molecular tools for the study of biofilm physiology. In Methods Enzymol San Diego Academic Press; 1999. p. 20-42.

Crawford JM, Kontnik R, and Clardy J. Regulating alternative lifestyles in entomopathogenic bacteria. Curr Biol 2010;20:69-74.

Felnagle EA, Barkei JJ, Park H, Podevels AM, McMahon MD, Drott DW, and Thomas MG. MbtH-like proteins as integral components of bacterial nonribosomal peptide synthetases. Biochemistry 2010;49:8815-8817.

Groll M, Schellenberg B, Bachmann AS, Archer CR, Huber R, Powell TK, Lindow S, Kaiser M, and Dudler R. A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. Nature 2008;452:755-758.

Imker HJ, Krahn D, Clerc J, Kaiser M, and Walsh CT. N-Acylation during glidobactin biosynthesis by the tridomain nonribosomal peptide synthetase module GlbF. Chemistry & Biology 2010;17:1077-1083.

Imker HJ, Walsh CT, and Wuest WM. SylC catalyzes ureido-bond formation during biosynthesis of the proteasome inhibitor syringolin A. J Am Chem Soc 2009;131:18263-+.

Krahn D, Ottmann C, and Kaiser M. The chemistry and biology of syringolins, glidobactins and cepafungins (syrbactins). Nat Prod Rep 2011;28:1854-1867.

Lautru S, Oves-Costales D, Pernodet JL, and Challis GL. MbtH-like protein-mediated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in Streptomyces coelicolor M145. Microbiology-Sgm 2007;153:1405-1412.

Mo Y-Y, and Gross DC. Plant signal molecules activate the *syrB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. J Bacteriol 1991;173:5784-5792.

Oka M, Nishiyama Y, Ohta S, Kamei H, Konishi M, Miyaki T, Oki T, and Kawaguchi H. Glidobactins A, B and C, new antitumor antibiotics. I. Production, isolation, chemical properties and biological activity. J Antibiot 1988a;41:1331-1337.

Oka M, Ohkuma H, Kamei H, Konishi M, Oki T, and Kawaguchi H. Glidobactins D, E, F, G and H; minor components of the antitumor antibiotic glidobactin. J Antibiot 1988b;41:1906-1909.

Prieto C, Garcia-Estrada C, Lorenzana D, and Martin JF. NRPSsp: non-ribosomal peptide synthase substrate predictor. Bioinformatics 2012;28:426-427.

Rackham EJ, Gruschow S, Ragab AE, Dickens S, and Goss RJM. Pacidamycin biosynthesis: Identification and heterologous expression of the first uridyl peptide antibiotic gene cluster. ChemBioChem 2010;11:1700-1709.

Ramel C, Tobler M, Meyer M, Bigler L, Ebert MO, Schellenberg B, and Dudler R. Biosynthesis of the proteasome inhibitor syringolin A: the ureido group joining two amino acids originates from bicarbonate. BMC Biochem 2009;10:26.

Schellenberg B, Bigler L, and Dudler R. Identification of genes involved in the biosynthesis of the cytotoxic compound glidobactin from a soil bacterium. Env Microbiol 2007;9:1640-1650.

Senior E, Bull AT, and Slater JH. Enzyme evolution in a microbial community growing on herbicide Dalapon. Nature 1976;263:476-479.

Staskawicz B, Dahlbeck D, Keen N, and Napoli C. Molecular characterization of cloned avirulence genes from race-0 and race-1 of *Pseudomonas syringae* pv. *glycinea*. J Bacteriol 1987;169:5789-5794.

Terui Y, Nishikawa J, Hinoo H, Kato T, and Shoji J. Structures of cepafungins I, II and III. J Antibiot 1990;43:788-795.

Titus JA, and Roundy CA. Improvement of glidobactin A production by Polyangium brachysporum. J Ind Microbiol 1990;6:215-218.

Wäspi U, Blanc D, Winkler T, Ruedi P, and Dudler R. Syringolin, a novel peptide elicitor from *Pseudomonas syringae* pv. *syringae* that induces resistance to *Pyricularia oryzae* in rice. Mol Plant-Microbe Interact 1998;11:727-733.

Wäspi U, Hassa P, Staempfli A, Molleyres L-P, Winkler T, and Dudler R. Identification and structure of a family of syringolin variants: Unusual cyclic peptides from *Pseudomonas syringae* pv. *syringae* that elicit defense responses in rice. Microbiol Res 1999;154:1-5.

Wuest WM, Krahn D, Kaiser M, and Walsh CT. Enzymatic timing and tailoring of macrolactamization in syringolin biosynthesis. Org Lett 2011;13:4518-4521.

Zhang WJ, Heemstra JR, Walsh CT, and Imker HJ. Activation of the Pacidamycin PacL Adenylation Domain by MbtH-like Proteins. Biochemistry 2010;49:9946-9947.

Figure legends

Figure 1

(A) Structural formula of glidobactin A. The compound is a tripeptide derivative with a 12-membered macrolactam ring and a fatty acid tail attached to the N-terminal threonine. **(B)** Alignment of the glidobactin synthetase gene cluster from K481-B101 (*glbA-H*) with the homologous gene cluster in *Ph. luminescens* ssp. *laumondii* TT01 (*plu1881-1877*). Vectored boxes indicate open reading frames (ORF's). Homologous genes are colored in black, whereas those that are absent in *Pll* TT01 are given in grey.

Figure 2

HPLC profiles of wild-type *Ps. putida* extract **(A)**, extract from *Ps. putida* expressing the *plu1881-1877* gene cluster **(B)**, and isolated glidobactin A standard **(C)**.

Figure 3

(A) Total ion UPLC chromatogram of methanolic extract of *Ps. putida* carrying the *plu1881-1877* gene cluster. **(B)** Magnified peak with retention time (RT) of 1.55 minutes that is expected to contain glidobactin. **(C)** UPLC-MS spectrum at RT 1.55 min. The two signals marked with asterisks correspond to protonated glidobactin ($C_{27}H_{45}N_4O$, m/z_{exp} 521.33327, $m/z_{calc.}$ 521.33336, 0.18 ppm error) and a glidobactin fragment generated in the ion source ($C_{11}H_{20}N_3O_3$, m/z_{calc} 242.14992, m/z_{exp} 242.14986, 0.24 ppm). The latter corresponds to the macrolactam ring of glidobactin without the extracyclic threonine and fatty acid tail.

Table 1. Prediction of amino acid specificity of NRPS modules encoded by *glbC/glbF* and *plu1880/plu1878*

Module	Position[a]										Observed	Predicted[b]
	235	236	239	278	299	301	322	330	331	517		
GlbF	D	F	W	N	I	G	M	V	H	K	Thr	Thr
Plu1878	D	F	W	N	I	G	M	V	H	K	Thr	Thr
GlbC-1	D	L	G	D	V	G	S	I	D	K	Lys	Lys
Plu1880-1	D	L	G	D	V	G	S	I	D	K	Lys	Lys
GlbC-2	D	V	G	W	I	A	G	I	V	K	Ala	-
Plu1880-2	D	V	G	W	I	T	G	I	V	K	Ala	-

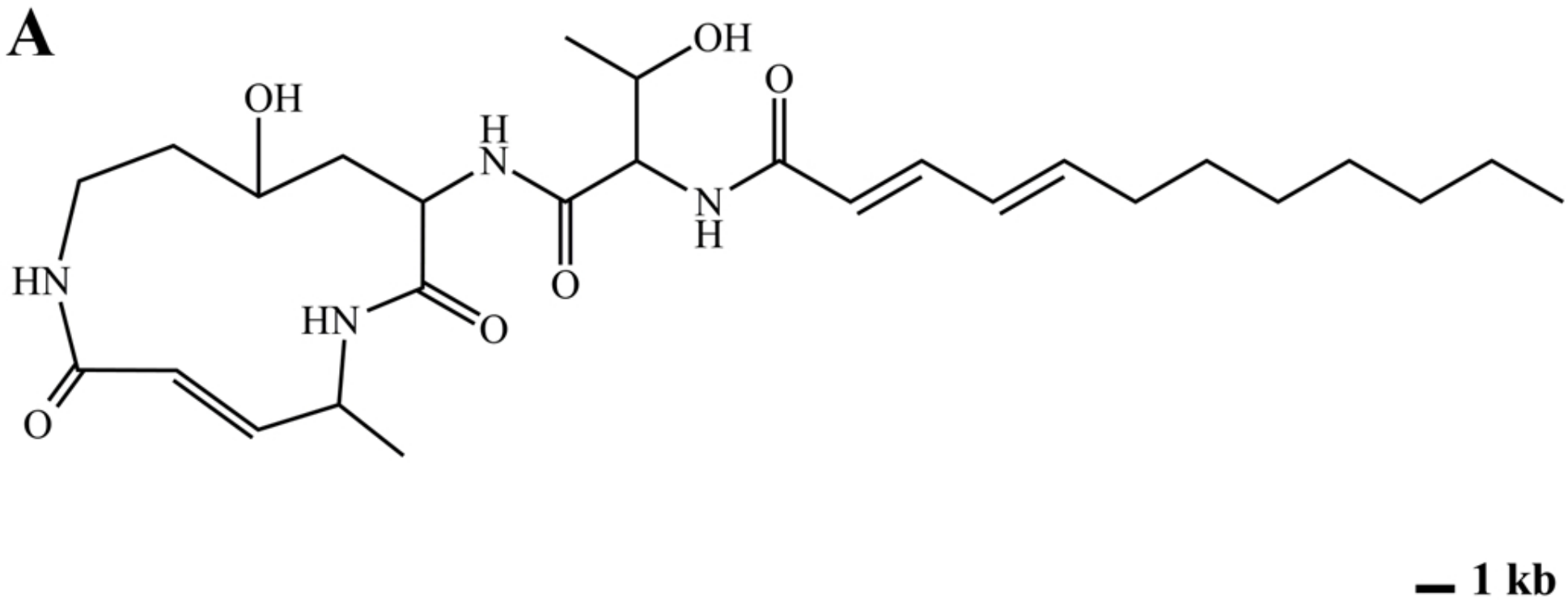
[a] Position of amino acid residues (given one-letter code) in the binding pocket of each of the NRPS modules as analyzed by NRPS-PKS software at http://www.nii.res.in/nrps-pks.html (Ansari et al., 2004).

[b] Amino acids (given in three-letter code) predicted to be activated by the respective NRPS module using the NRPSsp webtool at http://www.nrpssp.com (Prieto et al., 2012).

Table 2. List of oligonucleotides used

Name	Sequence	Overhang
Hind-Sal-Adapt-H	5'-AGCTTGGAAAGCTT	HindIII
Hind-Sal-Adapt-S	5'-TCGACAAGCTTTCCA	SalI
Xba-Bam-Adapt-X	5'-CTAGACATGTCAGGAG	XbaI
Xba-Bam-Adapt-B	5'-GATCCTCCTGACATGT	BamHI
plu1881_ f	5'-TATGCGTTGTACCACCTTCG	-
plu1881_r	5'-GCCGTTAGATCGATTTGGTG	-
plu1880_f	5'-ATAAACTCCAGTGCGCCATC	-
plu1880_r	5'-ATGTGGATTGTCCCTTCTGC	-
plu1877_f	5'-TTCAAATTTAAAGTAATCGCTGAGG	-
plu1877_r	5'-CGCAGAACTTCTTAGCTCAATG	-

Figure 1


A
OH
OH
O
H
N
N
H
HN
HN
O
O
O
1 kb
B
glb
A B
C
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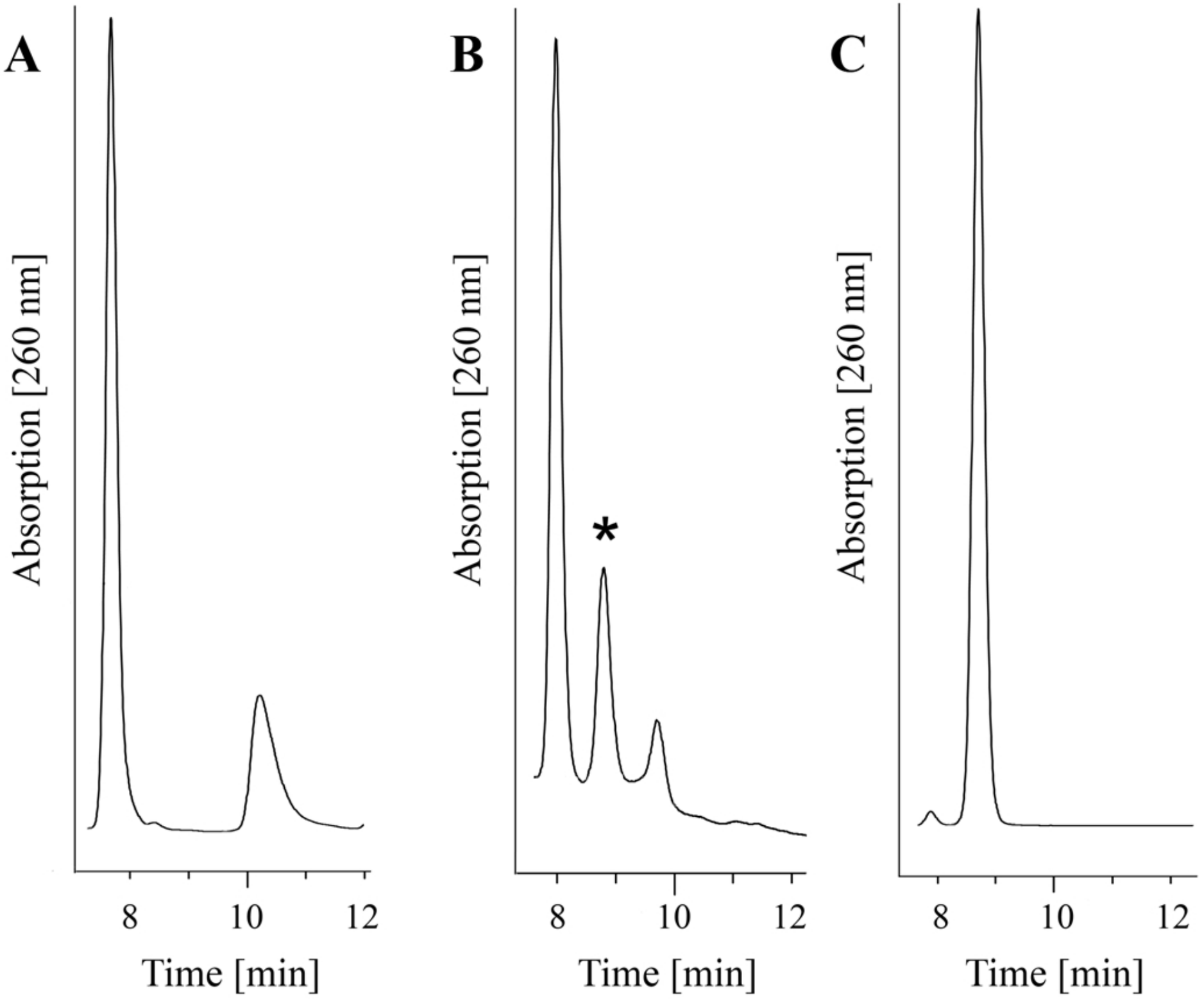
plu
1881
1880
1879 1878 1877
Pll TT01

Figure 2


A
B
C
Absorption [260 nm]
Absorption [260 nm]
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*
8
10
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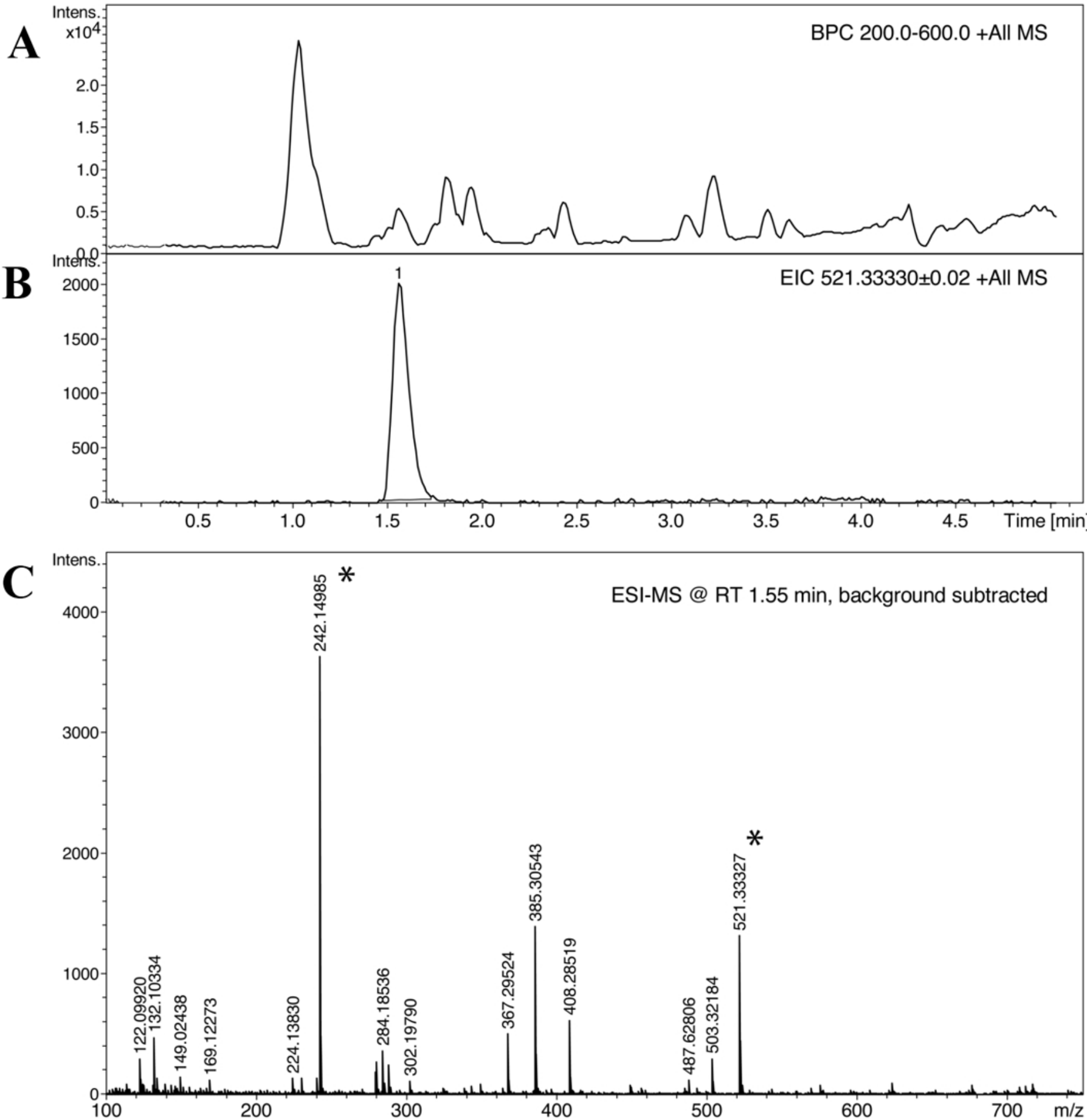
Figure 3


A
Intens.
x10^4
BPC 200.0-600.0 +All MS
B
Intens.
EIC 521.33330±0.02 +All MS
1
Time [min]
C
Intens.
ESI-MS @ RT 1.55 min, background subtracted
242.14985
385.30543
521.33327
122.09920
132.10334
149.02438
169.12273
224.13830
284.18536
302.19790
367.29524
408.28519
487.62806
503.32184
m/z